REMARKS

Applicants gratefully acknowledge the cordial personal interview accorded their undersigned representative by Examiner Aulakh on January 18, 2007. The following remarks are respectfully submitted to reflect the discussion which took place at the interview.

The indication of allowable subject matter in claims 25-46 is acknowledged with appreciation. By this amendment, claims 47 and 51 have been amended, and claims 50 and 52 have been canceled. Claims 1-49 and 51 are presented for further examination.

The rejection of claims 47-52 under 35 U.S.C. §112, first paragraph, is respectfully traversed with respect to the amended claims 47-49 and 51.

Although applicants cannot agree with the assertion that the terms "alleviating" or "inhibiting" imply completely curing the conditions recited in the claims, to reduce the issues in the application, the claims have been amended so that they no longer recite "alleviating" or "inhibiting" the recited conditions, but instead merely recite "treating" as proposed by the Examiner. The final office action acknowledges that the specification is enabling for treating pain, and accordingly, claims 47-49 are respectfully submitted to be allowable as now amended.

Claim 51 has been limited to treatment of epilepsy, stroke and ischemia. At the interview, Examiner Aulakh pointed out that the test data in the specification showed binding only to the glycine binding site of the NMDA receptor and requested evidence specifically the glycine binding site was implicated in the recited conditions being treated. Responsive to this request, submitted herewith as an evidentiary exhibit is a copy of an article by M. L. Mayer et al. entitled "A Physiologist's view of the N-Methyl-D-Aspartate Receptor: An Allosteric Ion Channel With Multiple Regulatory Sites" published in *Drug Development Research*, Volume 17, pages 263-280 (1989). This article discloses, *inter alia*, that:

> Drugs that competitively displace glycine from its binding site, but are without glycine-like activity, act as non-

> competitive NMDA receptor antagonists. The combination of these unusual physiological and pharmacological properties seems to have been exploited in the brain to allow the NMDA receptor to gate a variety of complex behaviours, including ... neuronal cell death in stroke and ischemia, and electrical excitability in epilepsy." (page 263, lines 1-6 from bottom)

The article thus discloses a direct role of the glycine binding site of the NMDA receptor in stroke, ischemia and epilepsy and evidences that the claimed compounds, which bind to the glycine binding site of the NMDA receptor, would be useful in the treatment of these conditions as described and claimed in the instant application. Accordingly, applicants respectfully submit that claim 51 as now amended is also fully enabled by the specification of the application. Reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, are accordingly respectfully requested.

The rejection of claims 1-24 under 35 U.S.C. §103(a) over Kobayashi and the rejection of claims 1-9, 11 and 15-24 under 35 U.S.C. §103(a) over Borrione are also respectfully traversed.

The final office action indicates that the rejections are based on the presumed obviousness of making salts of known compounds with bases. This presumes, however, that there is some reason why a person skilled in the art would be motivated to make such salts, such as the expectation that the salts would be useful for some beneficial use for which the prior art compounds were known to be useful. The problem with attempting to apply this logic in the instant case is that the cited references **do not disclose any utility** for the compounds they describe. Instead they merely describe synthesis reactions, but nothing more. It is well established in the law that if the prior art does not teach any specific or significant utility for the disclosed compounds, then the prior art is not sufficient to render structurally similar compounds *prima facie* obvious because there is no motivation for one of ordinary skill in the art to make the reference compounds, much less any structurally related compounds. *In re Stemniski*, 444 F.2d 581, 170 USPQ 343 (CCPA 1971). It follows that neither Kobayashi nor Borrione is sufficient to make out a *prima facie* case of

obviousness of the presently claimed salts. Reconsideration and withdrawal of the obviousness rejections are accordingly respectfully requested.

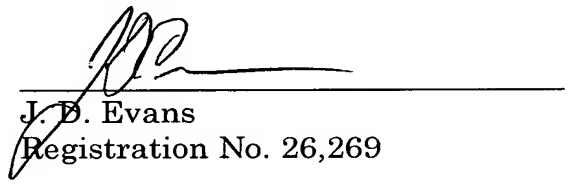
In view of the foregoing amendments and remarks, the application is respectfully submitted to be in condition for allowance, and prompt favorable action thereon is earnestly solicited.

If there are any questions regarding this Reply or the application in general, a telephone call to the undersigned at (202) 624-2845 would be appreciated since this should expedite the prosecution of the application for all concerned.

If necessary to effect a timely response, this paper should be considered as a petition for an Extension of Time sufficient to effect a timely response, and please charge any deficiency in fees or credit any overpayments to Deposit Account No. 05-1323 (Docket # 029310.53175US).

Respectfully submitted,

March 14, 2007



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Attachment: *Drug Development Research* 17:263-280 (1989)

Drug Development Research 17:263–280 (1989)

A Physiologist's View of the N-Methyl-D-Aspartate Receptor: An Allosteric Ion Channel With Multiple Regulatory Sites

Mark L. Mayer, Ladislav Vyklicky, Jr., and Evelyne Sernagor

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ABSTRACT

Mayer, M.L., L. Vyklicky, Jr., and E. Sernagor: A physiologist's view of the N-methyl-D-aspartate receptor: An allosteric ion channel with multiple regulatory sites. Drug Dev. Res. 17:263–280, 1989.

Whole-cell and single-channel recording techniques have provided a great deal of information about the nature of the N-methyl-D-aspartate (NMDA) receptor channel complex and are beginning to lead to quasimolecular models of drug action. Currently five ligand-binding domains are known to exist on the NMDA receptor channel complex: 1) an agonist-binding site for which structural analogues of L-glutamate have been developed as competitive antagonists; 2) a divalent cation-binding site within the ion channel pore, at which Mg binds to produce voltage-dependent channel block; 3) a divalent cation-binding site near the extracellular face of the membrane, at which Zn and Cd but not Mg bind to produce a voltage-independent block; 4) an anaesthetic binding site within the pore, at which ketamine, phencyclidine, MK-801, and desipramine bind with high affinity to produce voltage-dependent ion channel block of long duration; and 5) a regulatory site on the extracellular face of the membrane at which glycine binds to promote NMDA receptor channel activity. This potentiating action of glycine, to a large extent, reflects a block of fast desensitization, via an increase in the rate constant for return of desensitized NMDA receptors to their resting state. Drugs that competitively displace glycine from its binding site, but that are without glycine-like activity, act as noncompetitive NMDA receptor antagonists. The combination of these unusual physiological and pharmacological properties seems to have been exploited in the brain to allow the NMDA receptor to gate a variety of complex behaviours, including memory formation, neuronal cell death in stroke and ischemia, and electrical excitability in epilepsy. The existence of multiple ligand-binding

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sites on the NMDA receptor complex provides many potential avenues for the development of new classes of centrally active drugs.

Key words: patch clamp, ion channel block, desensitization, allosteric regulation

INTRODUCTION

Several receptors for excitatory amino acids are now known to occur in the mammalian central nervous system; the majority form receptor channel complexes and do not require the activation of second messenger systems to produce a change in neuronal excitability. Thus, binding of the appropriate amino acid agonist leads to a rapid increase in the frequency of ion channel opening; such responses are well suited to study by physiological techniques, especially patch clamp recording, and as a result, these are the best-characterized responses to excitatory amino acids. However, there is now good evidence for an additional type of L-glutamate receptor, activation of which produces a rise in intracellular calcium via a G-protein (guanosine nucleotide binding protein) and inositol 1,4,5-trisphosphate (IP_3) regulated second messenger cascade [e.g., Murphy and Miller, 1988]. Although this new receptor system is likely to play a major role in regulating nerve cell responses to L-glutamate, it seems likely that to fill their role as fast synaptic transmitters, the major action of excitatory amino acids occurs via direct control of ion channel gating rather than the activation of second messenger systems. In addition to the glutumate-evoked release of intracellular calcium stores triggered by IP_3 the ion flux through conventional excitatory amino acid receptor channels, or the depolarization this produces, can also lead to a rise in intracellular calcium ion activity, with consequent activation of many calcium-regulated proteins. Currently, several groups are exploring the role of calcium in the activation of various protein kinases that occurs following stimulation of excitatory amino acid receptors and the function this plays in synaptic plasticity.

Detailed studies on the action of excitatory amino acids were initiated by development of a three-receptor scheme by Watkins and his colleagues [Watkins and Evans, 1981], and several laboratories started to characterize the properties of the ion channels linked to kainate, N-methyl-D-aspartate (NMDA), and quisqualate receptors. After 5 years, a large number of advances have been made, but many problems remain to be solved. Pharmacological techniques have played a dominant role in this work, and the development of selective agonists and antagonists and new drugs continues to play a major role in this field. The key advances include recognition of the neuromodulatory role of NMDA receptors, with conventional fast excitatory synaptic transmission mediated by quisqualate or kainate receptors; the voltage-dependent regulation of NMDA receptors via ion channel block by Mg; the high calcium permeability of NMDA but not kainate or quisqualate receptor channels; and the allosteric regulation of NMDA receptors by nM concentrations of glycine. Linked to this molecular detail, there has emerged a widespread recognition of the role that NMDA receptors play in several complex processes including 1) aspects of synaptic plasticity such as memory in adults and development of the embryonic nervous system; 2) excitotoxicity, and the role this plays in mediating neuronal death during stroke and ischemia; and 3) the regulation of neural excitability and its changes during epilepsy. In this chapter, we will review the physiological studies on NMDA receptor channels that have led to the current frenzy of activity in this field; where relevant, we will try to emphasize the complex regulatory mechanisms that govern the activity of NMDA receptors.

ACTIVATION OF NMDA RECEPTORS

An extensive pharmacology has been developed for NMDA receptors, and at least 12 amino acids are known to have agonist activity; in addition, a large number of antagonists have

been developed [Watkins and Olverman, 1987]. However, in the nervous system it is likely that either L-glutamate, L-aspartate, or perhaps the sulfur amino acids L-homocysteate or L-homocysteine sulfinate act as endogenous ligands at NMDA receptors. To examine the potency of these substances at NMDA receptors, we have used a concentration-clamp technique for rapid application of amino acids to mouse hippocampal neurons grown in dissociated cell culture. Figure 1A shows that when recorded using the whole-cell patch-clamp technique, in conditions that minimize desensitization, with 5 mM Mg, 2 mM ATP, and 5 mM BAPTA (bis(*o*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) in the intracellular solution, and 3 μM glycine, 0 Mg, and 0.2 mM Ca in the extracellular solution, the dose-response curve for activation of NMDA receptors by L-glutamate is quite steep, suggesting cooperativity of agonist binding or channel opening. When analysed using the Hill equation, "n_H" the number of molecules of agonist required for receptor activation approaches a value of 1.7. The rank order of potency is L-glutamate > L-homocysteate > L-aspartate ≈ L-homocysteine sulfinate ≈ NMDA. The extremely low potency of the dipeptide N-acetyl-aspartylglutamate, which selectively activates NMDA receptors at mM concentrations, suggests that this substance is unlikely to act as a neurotransmitter [Westbrook et al., 1986].

The high potency of L-glutamate ($K_d \approx 3$ μM) has some interesting consequences for receptor function: the threshold for activation of NMDA receptors by L-glutamate is approximately 50 nM, and substantial inward currents can be evoked with 100 and 300 nM; because these concentrations are so low, diffusion of L-glutamate becomes a limiting factor in receptor activation, such that with a step concentration of 100 nM L-glutamate the current approaches steady state with a time constant of >50 ms. Recent studies on excitatory amino acid-mediated synaptic transmission have revealed dual component excitatory postsynaptic potentials (epsps), with fast components mediated by activation of non-NMDA receptors and slow components mediated via activation of NMDA receptors [Dale and Roberts, 1985; Forsythe and Westbrook, 1988]. This and the high potency of L-glutamate suggests several mechanisms by which NMDA receptors could be activated in vivo: 1) long-duration, NMDA-receptor-mediated epsps, lasting several hundred milliseconds, could be due to a transient release of relatively high concentrations of L-glutamate at synapses containing both kainate/quisqualate receptors and NMDA receptors (as discussed below, high concentrations of agonist are necessary to activate non-NMDA receptors), followed by prolonged NMDA receptor activation until the concentration of L-glutamate decreased to levels below 50 nM. 2) If NMDA receptors were located in the perijunctional membrane, close to synaptic junctions containing only kainate/quisqualate receptors, diffusion of L-glutamate along the dendritic arborization would be expected to generate slowly rising epsps, the duration of which could be limited by the transport of L-glutamate into cellular compartments by glial and neuronal uptake. Our results with concentration jump application of agonists suggest that if the concentration of L-glutamate was quite low, diffusion-limited activation of NMDA receptors could also contribute to the rise time of the excitatory postsynaptic current. 3) If there was a low (50–100 nM) concentration of L-glutamate in the extracellular fluid, a continuous background tone of NMDA receptor activation would exist; this would be expected to increase with neuronal activity, reflecting release of excitatory neurotransmitter into the extracellular medium.

Single-channel recording has revealed that as shown in Figure 1B, the ion channels linked to NMDA receptors have a moderately high conductance, around 50 picoSiemens (pS), and open in bursts of average length 8–10 msec with several openings separated by brief closings of microseconds duration [Jahr and Stevens, 1987; Ascher et al., 1988; Howe et al., 1988]. In addition, these channels show substate activity, with openings to 40, 30, 20, and 10 pS levels, with a large excess of open channel noise suggesting additional conformational states. To date, single-channel analysis of the dose-response curve for activation of NMDA receptors has not been attempted, but it is expected that the open probability will increase with agonist dose, as has been shown for invertebrate L-glutamate receptors [Cull-Candy et al., 1981]. The high

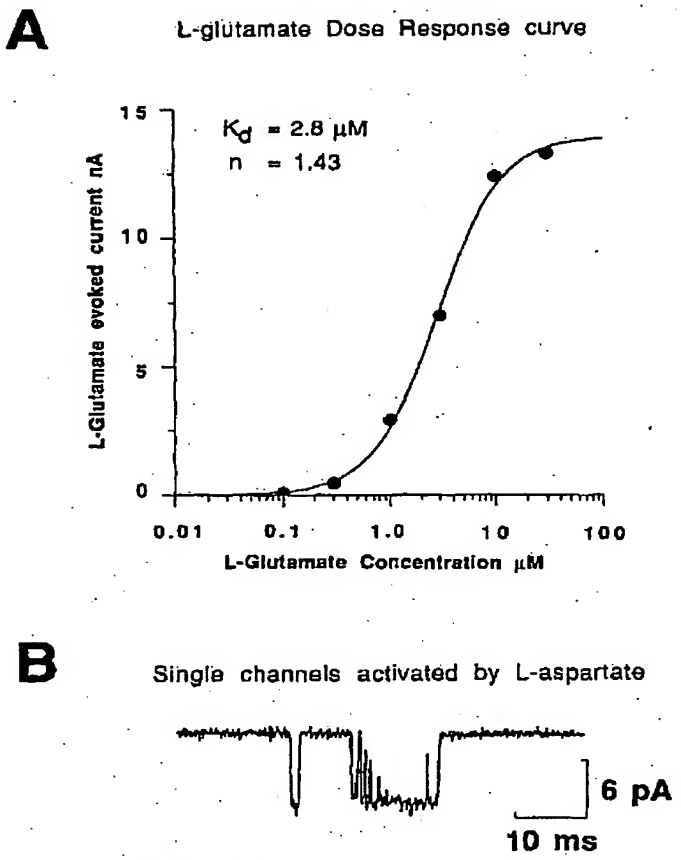


Fig. 1. Activation of NMDA receptors by L-glutamate and L-aspartate. A: Shows a dose-response curve for the response to L-glutamate recorded from a mouse hippocampal neuron in dissociated culture, using the tight-seal, whole-cell patch-clamp technique, and fitted with the Hill equation. L-glutamate was dissolved in a physiological solution with 0.2 mM Ca, 0 mM Mg, and 3 µM glycine and applied using a rapid perfusion technique capable of exchanging the extracellular solution with a time constant of ≤ 10 msec. The dose-response curve for L-aspartate was of similar shape, but shifted to the right reflecting a sevenfold lower affinity of L-aspartate for NMDA receptors. B: Shows high-resolution single-channel events evoked by L-aspartate and recorded from an outside out patch isolated from a rat cerebellar granule neuron in culture. NMDA receptor channels open in bursts with brief closures lasting on average only 50 µsec (A: Previously unpublished results of M.L. Mayer and L. Vyklicky) [B: reprinted with permission from Howe et al., 1988].

conductance of NMDA receptor channels has important implications for neuronal excitability, since the charge transfer during a single burst of openings is likely to be large enough to cause substantial changes in membrane potential, especially in structures of high impedance, such as dendritic spines.

In summary, our results show that several endogenous amino acids have a high potency for activation of NMDA receptors. The steep dose-response curve for activation of NMDA receptors and the occurrence of bursts of openings with very brief closed intervals suggest that the NMDA receptor is a doubly liganded receptor that must bind two molecules of agonist for full activation; in this model, brief closures during bursts could represent binding to and dissociation of agonist from a monoliganded receptor, although rapid transitions from a biliganded closed state to open states would produce similar responses.

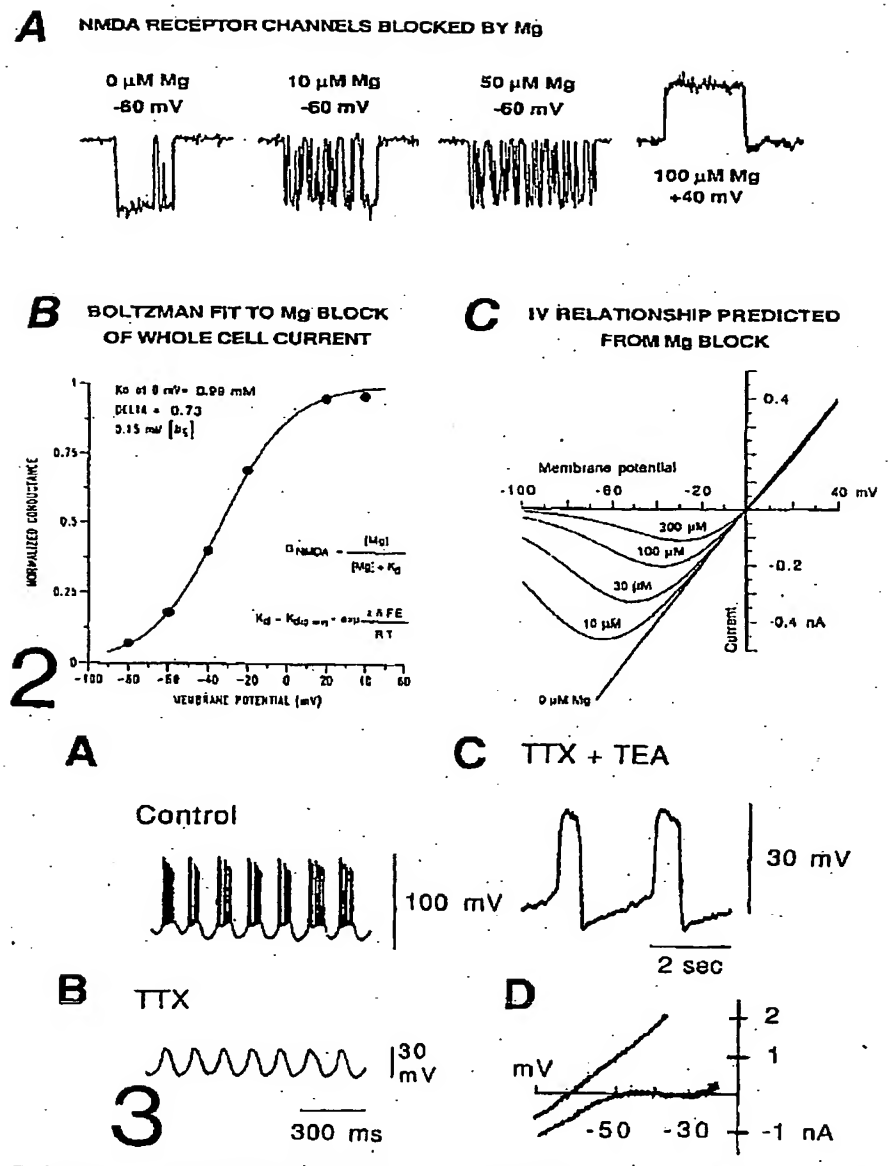
It is interesting also to consider briefly the specificity of action of the candidate neurotransmitter excitatory amino acids, that is, whether they activate other types of excitatory amino acid receptors in addition to NMDA receptors. We have found that, after blockade of NMDA receptor responses by removal of glycine and addition of 1 mM Mg, high concentrations of L-glutamate, L-homocysteate, and L-homocysteine sulfinate will also activate a fast desensitizing current identical with that activated selectively by low concentrations of quisqualate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). The potency of these endogenous amino acids was 2–3 orders of magnitude less for activation of quisqualate receptors than for activation of NMDA receptors; thus, highly selective activation of NMDA receptors is possible with low concentrations of any of these amino acids. Of interest, L-aspartic acid was virtually without activity at quisqualate receptors such that 1–3 mM concentrations produced virtually no response when NMDA receptor responses were blocked. The synaptic release of L-aspartic acid would, therefore, produce pure slow epsps.

REGULATION OF NMDA RECEPTOR ACTIVITY BY MAGNESIUM

The extracellular fluid of the mammalian CNS contains a millimolar concentration of magnesium; like calcium, the concentration of magnesium is tightly regulated, to such an extent that when the plasma concentration of magnesium in human subjects is raised artificially to a level sufficient to cause respiratory paralysis owing to block of synaptic transmission at the neuromuscular junction, consciousness and central synaptic transmission are unimpaired [Somjen et al., 1966]. Also, prolonged periods on a magnesium-free diet fail to produce a fall in the concentration of Mg in the cerebrospinal fluid, suggesting that it is highly important for the brain to keep the concentration of Mg regulated at a physiological value [Fishman, 1980]. One central neurotransmitter receptor highly sensitive to changes in the extracellular concentration of Mg is that activated by NMDA. Many laboratories have now confirmed and extended the original finding that sub-millimolar concentrations of Mg have a powerful antagonist action on NMDA receptor responses [Ault et al., 1980]. It is perhaps fortunate that early studies that led to the development of selective NMDA receptor antagonists such as 2-amino-5-phosphonovaleric acid (AP5) were performed using as an assay system an isolated amphibian spinal cord [Watkins and Evans, 1981], since for traditional reasons, amphibian Ringer's solution is magnesium free; without this fortuitous accident, it is likely that the excitatory potency of NMDA would have been so low as to preclude the search for NMDA antagonists.

It is now well known that magnesium ions enter and block NMDA receptor channels. At the single-channel level, the blocking effect of Mg is seen as a high frequency flicker, as Mg ions rapidly enter, block, and then dissociate from NMDA receptor channels [Nowak et al., 1984; Ascher and Nowak, 1988]. As shown in Figure 2A, this blocking action only occurs when the membrane potential is held at a negative voltage. This is because the Mg-binding site is deep within the NMDA receptor channel complex; as a result the dissociation constant for Mg changes with voltage, such that the affinity for Mg increases with hyperpolarization. As shown in Figure 2B, the dissociation constant for Mg changes e-fold per 17.6 mV, according to the following model:

$$Kd = K_{(o)} * \exp\left(\frac{z\,\delta FVm}{RT}\right)$$



in which $K_{(o)}$ (the equilibrium dissociation constant of Mg at a membrane potential of zero mV) is 0.99 mM; δ (the fraction of the membrane electric field at the Mg-binding site) is 0.73; Vm is the membrane potential, and z, F, R, and T have their usual meaning.

This effect of magnesium has many interesting and highly important physiological consequences. Because responses to excitatory amino acids are produced by activation of cation-selective channels equally permeable to Na and K, responses to excitatory amino acids have an equilibrium potential close to 0 mV [Mayer and Westbrook, 1984]. For conventional, voltage-insensitive ion channels of the type activated by kainate and quisqualate, stepping the membrane potential away from the equilibrium potential should, to a first approximation, produce progressively larger responses, such that a plot of membrane potential vs. current response would be linear, with the equilibrium potential as its origin. However, for responses to NMDA, the effect of membrane potential on the dissociation constant for Mg has an additional effect: at inside positive membrane potentials the dissociation constant for Mg becomes so weak that Mg block produces little or no reduction in the amplitude of the response to NMDA; but as the membrane potential is hyperpolarized, the dissociation constant for Mg shifts to higher affinity, such that at inside negative potentials, there is a strong block of responses to NMDA.

Because membrane potential hyperpolarization has two opposing effects on the amplitude of responses to NMDA—an increase in driving force for inward current, producing larger responses, and an increase in the blocking action of Mg, producing smaller responses—the relationship between the membrane potential and the amplitude of responses to NMDA is complex but can be predicted on the basis of the voltage-dependent binding curve for Mg shown in Figure 2B. It can be calculated that with 1 mM Mg, the maximum inward current through NMDA receptor channels will be recorded at approximately −30 mV, as shown in Figure 2C, and that further depolarization will reduce the amplitude of the response, reflecting a reduction in driving force for Na influx, while further hyperpolarization will reduce the amplitude of the response because of the increase in the blocking action of Mg. As a result, the shape of the current voltage (IV) curve for NMDA receptor responses varies with $[Mg]_o$ in a highly characteristic manner, as shown in Figure 2C; in particular the IV relationship is

Fig. 2. Voltage-dependent block of NMDA receptor channels by Mg. A: Shows NMDA receptor single-channel activity recorded from an outside out patch at either −60 or +40 mV, and exposed to 0, 10, 50, or 100 μM Mg. At −60 mV, 10 and 50 μM Mg produce high-frequency bursts as Mg enters and blocks the NMDA receptor channel; at +40 mV Mg, 100 μM, has no blocking action. B: Shows an analysis of the antagonism by 150 μM Mg of whole cell responses to NMDA, recorded over the membrane potential range −80 to +40 mV, and fitted assuming that when 1 molecule of Mg binds to the NMDA receptor channel, ion permeation is blocked. The K_d for Mg is assumed to change exponentially with the membrane potential, decreasing e-fold per 17.6 mV hyperpolarization, from a value of 0.99 mM at zero mV. C: Shows whole-cell current-voltage relationships predicted using the binding model described in B, with 0, 10, 30, 100, and 300 μM extracellular Mg; note the negative slope conductance characteristic of the IV relationship. The peak inward current occurs at progressively more depolarized membrane potentials as $[Mg]_o$ is increased [A: reprinted with permission from Ascher and Nowak, 1988] (B: Reprinted with permission from Mayer et al., 1989a; C: Previously unpublished calculation of M.L. Mayer).

Fig. 3. Burst discharge of action potentials evoked by NMDA applied to a cat neocortical neuron in a brain slice prepared from the area of the lateral cruciate sulcus. A: Shows the burst firing pattern evoked by NMDA in extracellular solution of physiological composition; after block of sodium current evoked action potentials with tetrodotoxin (TTX), application of NMDA evokes oscillatory membrane potential depolarizations (B), which triggered the action potential discharge shown in A. Exaggerated bistable membrane potential behaviour (C) is recorded after reduction of potassium currents with tetraethylammonium (TEA), when the membrane potential jumps between −50 and −30 mV. The current voltage plot shown in D was obtained under voltage clamp, and illustrates the near zero slope conductance recorded between −50 and −30 mV, centered around zero membrane current, which underlies the bistable behaviour shown in C [reprinted with permission from Flatman et al., 1983].

linear in Mg-free solution, and the membrane potential at which peak inward current is recorded shifts to more positive values as $[Mg]_o$ is increased. The total amount of inward current recorded at all membrane potentials decreases as $[Mg]_o$ rises. The reduction in response to NMDA seen with hyperpolarization negative to −30 mV is referred to as a region of negative slope conductance. It should be noted that even at the resting potential, say −60 mV, the response to NMDA will not be completely blocked. This is often overlooked and has given rise to the incorrect idea that NMDA receptor channels are only active once the membrane potential is depolarized.

The blocking action of magnesium has profound consequences for the action of NMDA on neuronal excitability, which ultimately explains the potent action of NMDA receptor antagonists as anticonvulsants. The increase in amplitude of responses mediated via activation of NMDA receptors that occurs during depolarization close to the resting potential makes the nerve cell membrane electrically unstable; the effect is like positive feedback: activation of NMDA receptors produces a small depolarization, which relieves Mg block, which produces a larger depolarization, which further relieves Mg block, which produces a still larger depolarization, and so on. Of course, potassium channels in the nerve cell membrane are also opened by membrane potential depolarization and normally produce a hyperpolarizing current to reduce the excitatory influence of the NMDA-activated response.

However, an unusual situation can occur when the negative slope conductance of the response to NMDA exactly balances the usually positive slope conductance because of opening of potassium channels in the nerve cell membrane [Flatman et al., 1983]: the membrane potential now has two equally stable values and will exhibit bistable behaviour as jumps between these two values occur, as shown in Figure 3. This behaviour produces periods of quiescence separated by intense bursts of activity, very similar to convulsant activity in uncontrolled epilepsy. The fact that NMDA receptor antagonists are powerful anticonvulsants suggests that the activation of NMDA receptors does contribute to neuronal discharge during epilepsy, although whether bistable behaviour of the type described, or a more modest activation of NMDA receptor-mediated voltage-dependent excitatory synaptic responses underlies the epileptic event remains to be determined.

CALCIUM FLUX THROUGH NMDA RECEPTORS

Both NMDA and kainate-quisqualate receptor responses are mediated by activation of cation-selective ion channels, which are permeable to small monovalent cations. However, NMDA receptor channels also have a high permeability to calcium, and this has important consequences for neuronal function, since the activation of several ion channels, and many second messenger systems, is controlled by the cytoplasmic calcium ion concentration.

Direct evidence for calcium flux through NMDA receptor channels was obtained in experiments in which a calcium-sensitive dye, Arsenazo III, was introduced into the cell body of spinal cord neurons, which were voltage clamped at −60 mV to prevent NMDA-induced depolarization, and the subsequent activation of voltage-dependent calcium channels [MacDermott et al., 1986]. As shown in Figure 4A, responses to NMDA were accompanied by wavelength-dependent changes in transmittance, consistent with the known spectral sensitivity of Arsenazo III to changes in calcium ion concentration. Comparable responses to kainate and quisqualate were accompanied by only small increases in $[Ca]_i$, provided the membrane potential was voltage clamped to prevent activation of calcium channels. By measuring the equilibrium potential of responses to NMDA, kainate, and quisqualate over a wide range of extracellular calcium ion concentration, a quantitative estimate of the relative permeability of excitatory amino acid-activated ion channels to Na and Ca was obtained [Mayer and Westbrook, 1987]; NMDA receptor channels behaved as though they were 10.6 times more permeable to Ca than to Na, while for kainate and quisqualate the ratio was 0.15, making

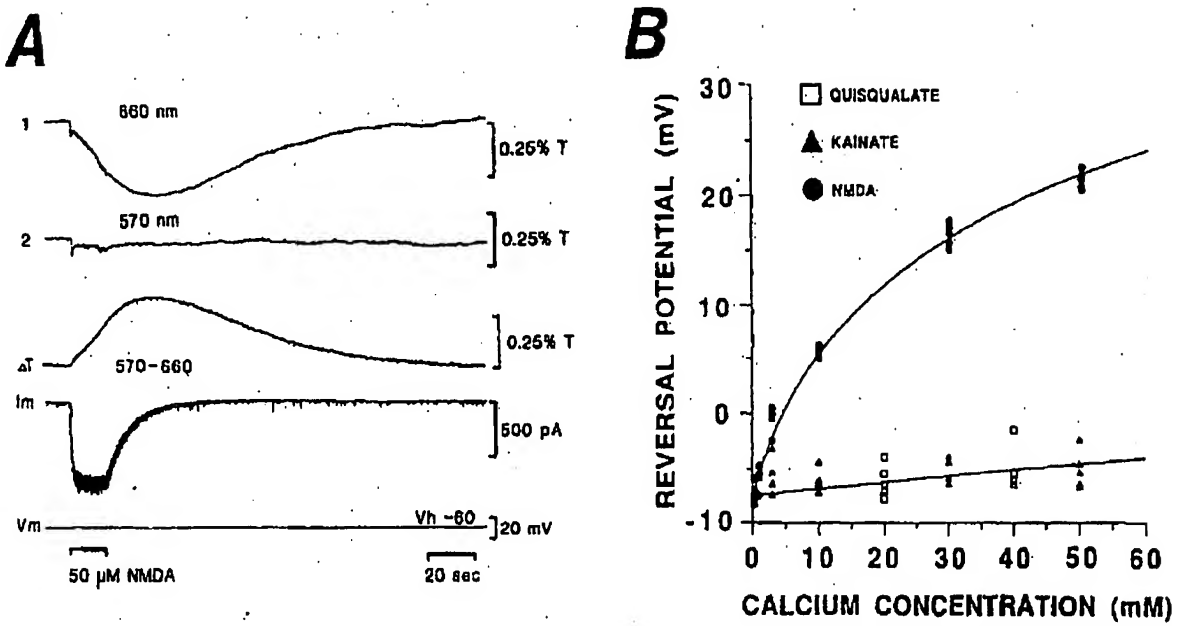


Fig. 4. Calcium flux through NMDA receptor channels. **A:** Shows combined optical and voltage-clamp recording from a mouse spinal cord neuron in dissociated culture loaded with the calcium indicator dye Arsenazo III. Application of NMDA produces a large inward current response at −60 mV, accompanied by a decrease in transmittance at 660 (1) but not 570 (2) nM; subtraction of this wavelength pair improves the signal-to-noise ratio (ΔT), and the resultant signal illustrates the rise in intracellular calcium ion activity evoked by NMDA. **B:** Shows a plot of the reversal potential for the response of mouse hippocampal neurons to quisqualate, kainate, and NMDA, ploted as a function of $[Ca]_o$, and fitted with the extended Goldman, Hodgkin, Katz constant field equation, assuming that for NMDA $P_{Ca}/P_{Na} = 10.6$ [A: reprinted with permission from MacDermott et al., 1986; B: reprinted from Mayer and Westbrook, 1987].

NMDA receptor channels 70 times more permeable to Ca than kainate or quisqualate receptor channels. Because the extracellular concentration of calcium is quite low, say 2 mM, while sodium is present at approximately 140 mM, calcium flux through NMDA receptor channels can be calculated to be approximately 12% of the inward current at −60 mV. This analysis was made with many assumptions concerning the nature of the NMDA receptor channel permeation process, and it is likely that these are gross oversimplifications of the actual mechanism. However, until data can be obtained to support a more realistic model, with multiple ion-binding sites and perhaps a vestibule with fixed negative charges, our results, fitted using extended constant field theory, provide a qualitative basis on which to compare the behaviour of NMDA receptor channels with other cation-selective channels.

Important in understanding the behaviour and consequences of NMDA receptor activation is the spatial location of NMDA receptors within the nerve cell membrane, and additional information is needed concerning the cellular localization of NMDA receptors. For example, if NMDA receptor channels were located on dendritic spines, agonist-triggered calcium influx could be quite compartmentalized. This would have two important consequences: 1) membrane and cytoplasmic proteins at individual spines could be selectively modified via activation of Ca-dependent kinases; and 2) the lower buffering capacity of a small compartment could result in a much higher elevation of $[Ca]_i$ than if NMDA receptors were distributed only on larger processes. Even in larger cellular compartments, the relatively slow diffusion coefficient of Ca, which is in part due to binding of Ca to calcium-binding proteins, provides a mechanism whereby the spatial and temporal Ca gradient following a transient calcium influx can remain quite localized for many 100s of msec, providing a mechanism for selective activation of second messenger systems.

THE ACTION OF GLYCINE AND GLYCINE ANTAGONISTS ON NMDA RECEPTORS

Steady-state responses to NMDA show strong potentiation by nM concentrations of glycine, D-alanine, and D-serine. This effect occurs with no change in single-channel conductance and very little change in mean open time, suggesting that NMDA receptors have a high-affinity binding site for glycine, which allosterically regulates the frequency of channel activation [Johnson and Ascher, 1987].

We have found that with fast application techniques, responses to NMDA show strong desensitization, and that the degree of desensitization varies with the concentration of glycine, such that as the glycine-binding site is titrated, NMDA receptor desensitization is reduced and eventually blocked when the glycine-binding site becomes saturated (Figure 5). The time constant of NMDA receptor desensitization is quite fast, close to 200 msec, and does not vary with either the concentration of NMDA or glycine; rather, as the glycine concentration is increased, the rate constant of recovery from desensitization becomes faster (Mayer et al., 1989b). Since fast application techniques are required to record the peak current response to NMDA, before desensitization occurs, responses to NMDA recorded using slower application techniques reflect an equilibrium of nondesensitized and desensitized receptors, and the potentiating action of glycine to a large extent actually reflects block of desensitization. To our knowledge, these results are the first description of the mechanism of allosteric regulation of an ion channel, which have a basis derived from kinetic analysis of receptor channel activation. It is at first surprising that the action of glycine, which strongly potentiates NMDA responses measured at equilibrium, is due to a reduction of fast desensitization, since on first inspection, it would be expected that allosteric modulators that increase the frequency of ion channel activation would also enhance desensitization, since desensitization and activation are usually conceptualized as linked processes.

We have recently found that the nonselective amino acid antagonist kynurenic acid has a dual mechanism of action on NMDA receptors, reflecting competitive antagonism at both the

agonist- and glycine-binding sites. Both effects occur with no change in mean open time or single-channel conductance, estimated from spectral analysis of agonist-activated currents [Mayer et al., 1988]. Recent experiments at the laboratories of Merck, Sharp & Dhme have shown that the 7-Cl derivative of kynurenic acid has a much higher affinity (0.56 μM) for the glycine-binding site than native kynurenic acid (41 μM) and has only a low affiity for the agonist-recognition site on the NMDA receptor complex (169 μM) such that with low concentrations of 7-Cl-kynurenic acid, selective NMDA receptor antagonism can b produced solely via block of the modulatory action of glycine [Kemp et al., 1988].

GROUP IIB CATIONS AND DISSOCIATIVE ANAESTHETICS ACT AT TWO ADDITIONAL SITES TO REGULATE NMDA RECEPTOR ACTIVITY

In addition to the novel noncompetitive antagonists described above, which block the action of glycine, a battery of selective, competitive antagonists, which are structurall analogues of L-glutamate have been developed as NMDA receptor antagonists. Severall divalent cations, including cobalt, nickel, and manganese, also act as NMDA antagonists, via an action at the Mg-binding site within the ion channel. Recent experiments have revealed an additional two sites for pharmacological manipulation of NMDA receptor activity. Low micromolar concentrations of zinc and cadmium also block responses to NMDA, as shown in Figure 6, but with minimal voltage dependence, suggesting that these ions bind to an additional site on the NMDA receptor channel complex that is situated close to the extracellular face of the membrane [Mayer et al., 1989a]. The action of zinc and cadmium is selective for NMDA receptors and is quite potent, with the K_d estimated at 13 and 48 μM for zinc and cadmium, respectively. No reduction of responses to kainate and quisqualate occurs unless micromolar concentrations are applied.

There are several mechanisms that can be imagined for reduction of NMDA receptor activity, including competitive antagonism at either the agonist recognition site or the glycine-binding site and channel block. However, the action of zinc and cadmium as NMDA antagonists is noncompetitive and does not vary with the concentration of NMDA, in contrast to what would be expected for both competitive antagonists and ion channel blockers, since the latter would be expected to show uncompetitive kinetics. Zinc antagonism occurs with only a small change in mean open time and conductance, and single-channel recording experiments show that the major effect is a reduction in the frequency of channel activation, similar to the effect that would be produced by a glycine antagonist. Despite this, zinc is unlikely to act as a competitive glycine antagonist because there is only a small reduction in zinc potency, as the glycine concentration is raised over the range 100 nM to 0.1 mM. It is possible that zinc antagonism of NMDA receptor responses reflects either an indirect effect of zinc on glycine binding or, more likely, a functional antagonism of the glycine-mediated positive allosteric effect via an independent negative allosteric effect of zinc on ion channel gating.

A second site for modulation of NMDA receptor activity was discovered by Lodge and his associates, who observed that ketamine, phencyclidine, and related drugs classified as σ-opiates were potent, selective NMDA antagonists, with little action on responses evoked by kainate or quisqualate [Anis et al., 1983]. This observation was extended to show that the action of ketamine was highly voltage dependent, showed use-dependent block, and required the presence of agonist for recovery from antagonism; although the onset of and recovery from antagonism was slow, fluctuation analysis revealed a reduction in single-channel open time during ketamine antagonism, suggesting rapid block of individual receptor channels [Honey et al., 1985; Mayer et al., 1988]. Together, these results suggested that ketamine acted as a channel blocker, similar in action to Mg, but more potent; in addition, use-dependence for the onset and recovery from antagonism suggested that the NMDA receptor channel had to be activated for ketamine to gain access to its binding site, and that the channel could close with

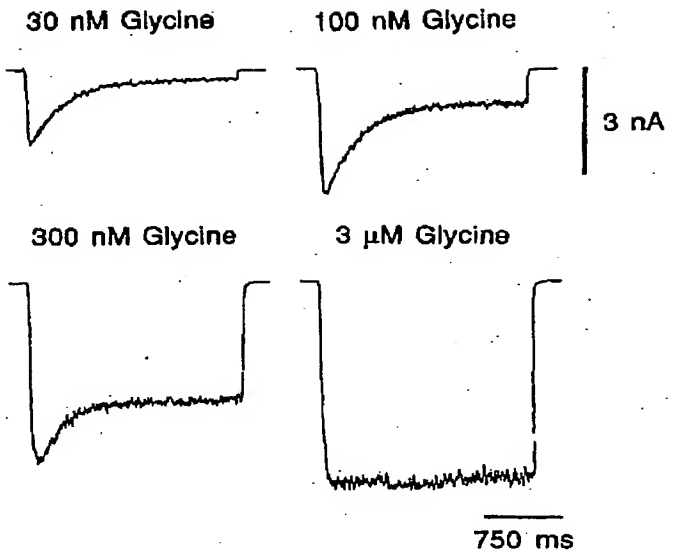


Fig. 5. Glycine blocks fast desensitization at NMDA receptors. The traces show whole-cell responses to rapid applications of 100 μM NMDA at different extracellular glycine concentrations, following a 15-sec preequilibration with glycine. The responses recorded with 30, 100, and 300 nM glycine were recorded from one cell; the response to 3 μM glycine was recorded from a second cell and has been scaled by matching the amplitude of the response of both cells to NMDA applied with 300 nM glycine (scaling factor × 1.122). The potentiation of the NMDA response recorded on raising [gly] from 30 nM to 3 μM is much larger when measured at steady state (23.4) than the increase in amplitude measured at the peak response to NMDA (2.83). This reflects a dramatic reduction of desensitization by glycine.

ketamine still in the channel, trapping ketamine molecules within the NMDA receptor channel complex.

Recently, the anticonvulsant MK-801 has been shown to have a similar action to ketamine, but acts with much higher potency [Huettner and Bean, 1988]. Both binding studies and physiological experiments give similar estimates for the dissociation constants for this family of drugs, which span several orders of magnitude: the values are ketamine 5–10 μM; phencyclidine 0.5–2 μM; MK-801 10–30 nM. The extremely high potency of MK-801 reflects very slow dissociation from the receptor channel complex; as shown in Figure 7A, this rate constant is quite voltage dependent, and increases e-fold per 25 mV depolarization, although this is likely to be only an approximate figure given the slow time constant of recovery at −70 mV (92 min) and the extreme difficulty of measuring such slow processes while recording from single nerve cells using traditional electrophysiological techniques. However, slow voltage-dependent dissociation does appear to be a characteristic feature of the action of antagonists, which bind to the ketamine/MK-801-binding site on NMDA receptors, and this is likely to provide a useful indicator in assigning a site of action to novel NMDA antagonists.

Recent experiments on the pharmacology of NMDA receptors have shown that the tricyclic antidepressant desmethylimipramine inhibits the binding of [^{3}H] MK-801 to the

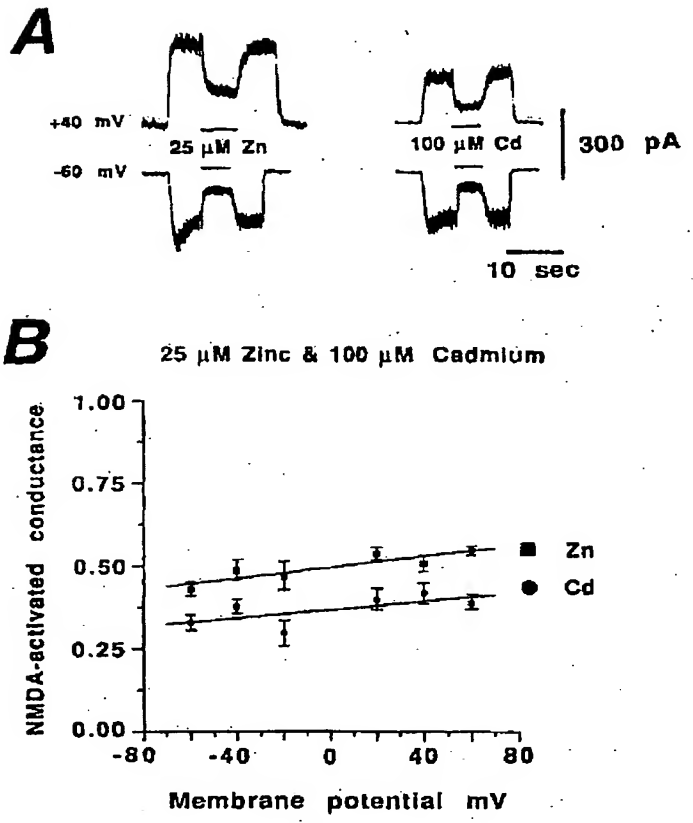


Fig. 6. Zinc block of NMDA responses is not highly voltage dependent. A: Shows antagonism of responses to NMDA by 25 μM Zn and 100 μM Cd and recorded at −60 and +40 mV. B: Shows analysis of similar results assuming that the binding of zinc and cadmium is weakly voltage dependent. In contrast to the highly voltage dependent block produced by Mg, a several hundred mV change in membrane potential is required to produce an e-fold change in the K_d for zinc block of NMDA responses, suggesting that the binding site for Zn is close to the extracellular face of the membrane [reprinted from Mayer et al., 1989a].

NMDA receptor complex and in functional assays blocks the NMDA-mediated calcium influx into cultured neocortical neurons [Reynolds and Miller, 1988]. Kinetic analysis of the effects of desmethylimipramine on the binding of MK-801 revealed a decrease in the off-rate constant of dissociation of MK-801 from the NMDA receptor complex, an effect similar to that observed with zinc. Since both zinc and desmethylimipramine act as noncompetitive NMDA receptor antagonists, it was suggested that they share a common site of action. However, in physiological experiments, we have found that the block of NMDA receptor responses by desmethylimipramine has many features that are characteristic of the action of ketamine and

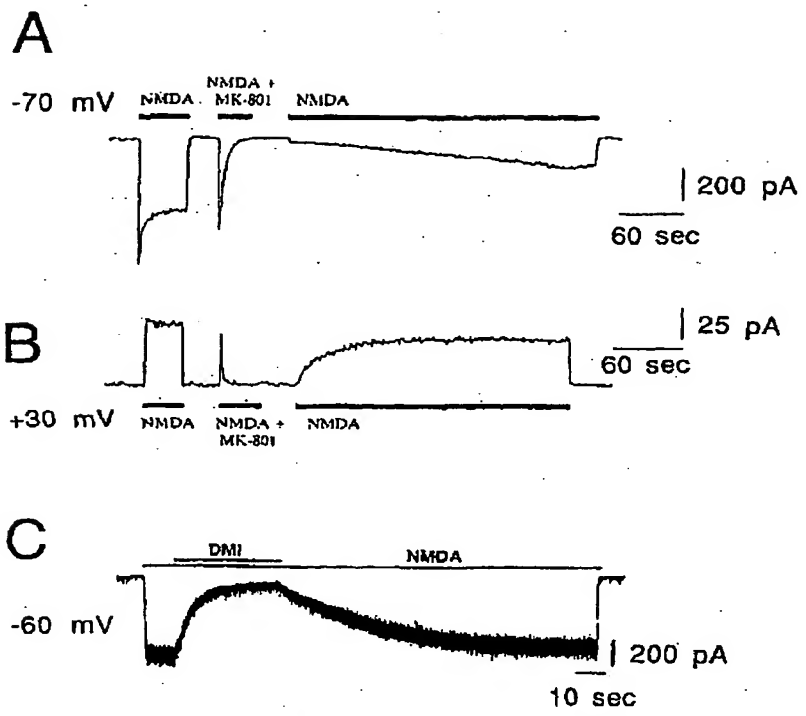


Fig. 7. Slow dissociation of MK-801 and desmethylimipramine from NMDA receptor channels. A, B: Show antagonism by 10 μM MK-801 of NMDA receptor responses recorded from rat visual cortex neurons in culture; the off-rate constant for dissociation of MK-801 is highly voltage dependent, and is much faster at +30 than at −70 mV. C: Shows antagonism by 50 μM desmethylimipramine (DMI) and slow recovery of the response to NMDA recorded from a mouse hippocampal neuron in culture; the off-rate constant for dissociation of DMI showed a similar voltage-dependent increase on membrane potential depolarization (A,B: reprinted with permission from Huettner and Bean, 1988] (C: reprinted with permission from Sernagor et al., 1989).

MK-801. The onset of antagonism of NMDA receptor responses by desmethylimipramine shows use-dependent kinetics, and as shown in Figure 7B, there is a prolonged recovery phase from desmethylimipramine antagonism similar to that seen with ketamine and MK-801. The equilibrium block of NMDA receptor responses by desmethylimpramine is highly voltage dependent, increases with hyperpolarization, and behaves as if the dissociation constant for binding of desmethylimipramine changes e-fold per 36 mV change in membrane potential. Although desmethylimipramine is quite potent, and at −60 mV the dissociation constant of 9.8 μM is close to that measured for ketamine (5–10 μM), it is unlikely that the action of desmethylimipramine at NMDA receptors is important for its therapeutic action, which occurs at much lower plasma concentrations. The above results suggest that the major action of tricyclic depressants on NMDA receptors occurs via channel block and not via a zinc-like effect, since the latter is effectively voltage independent. It seems plausible that the effect of

desmethylimipramine on the MK-801 off-rate constant measured in binding studies could be due to steric hindrance if binding of desmethylimipramine to the NMDA receptor complex trapped MK-801 within the channel. Our analysis of the voltage dependence of the action of desmethylimipramine showed slightly weaker voltage dependence ($\delta = 0.71$) than observed with ketamine ($\delta = 1.0$), which would be consistent with a shallower binding site for desmethylimipramine.

THE NMDA RECEPTOR IS A COMPLEX ION CHANNEL WITH MULTIPLE SITES FOR DRUG ACTION

Many exciting molecular details are emerging concerning the structural domains of the NMDA receptor channel complex (Fig. 8). The above review clearly points to the existence of several distinct sites at which pharmacological agents can act to regulate the activity of NMDA receptors; these include 1) an agonist-binding site at which structural analogues of L-glutamate act as competitive antagonists; 2) a glycine-binding site, which modulates NMDA receptor activity, and at which kynurenic acid and its derivatives bind and competitively displace glycine, to produce noncompetitive antagonism of responses to NMDA; 3) a binding site for dissociative anesthetics such as ketamine, phencyclidine, and MK-801, which is deep within the ion channel; 4) a binding site, also within the channel, for divalent cations, which at negative membrane potentials has high affinity for Mg, Ni, Co, and Mn; 5) a binding site for zinc and cadmium, which is located near the extracellular face of the membrane. At present, it seems premature to assign separate binding sites to each of the organic cations that are potent NMDA antagonists, since all have common structural features, most notably an amine group or derivative that is charged at physiological pH and linked to bulky unsaturated ring structures; however, subtle differences in the behaviour of desmethylimipramine and MK-801 in binding assays and in physiological experiments suggest that eventually it may not be possible to accommodate the action of all these drugs within a single binding site model.

Although at first inspection there would seem to be only one consequence of NMDA receptor antagonism—shut down of the many complex cellular behaviours regulated by NMDA receptor activity—there are good reasons for believing that the availability of a battery of pharmacologically active substances acting at NMDA receptors could eventually lead to the development of drugs with quite specific effects on individual NMDA receptor-regulated behaviours. There are two reasons for this. First, the different pharmacokinetics of competitive antagonism, noncompetitive antagonism, and use-dependent ion channel block already make it possible in principle to design drugs that will selectively block NMDA receptor responses only during periods of intense activity (use-dependent antagonists, with slow dissociation), or that will be insensitive to changes in excitatory amino acid receptor tone (noncompetitive antagonists such as 7-Cl-kynurenic acid, which act at the glycine-binding site). Second, recent structural data on $GABA_A$ receptor channel complexes obtained using molecular biological approaches have revealed the occurrence of multiple GABA receptor subtypes, with subtle differences in their agonist affinity, and probably their pharmacological sensitivity, and which are selectively expressed in different areas of the brain; the logical extension of this finding is the design of drugs that will modulate GABA receptors in one part of the brain, and not in others. Although there are no structural data available at present for excitatory amino acid receptor channel complexes, it seems quite reasonable to look forward to the discovery of multiple NMDA receptor subtypes and the development of subtype specific antagonists.

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The group's research program on excitatory amino acids was initated jointly by M.L. Mayer and G.L. Westbrook during the period 1983–1987, and the work described here is a

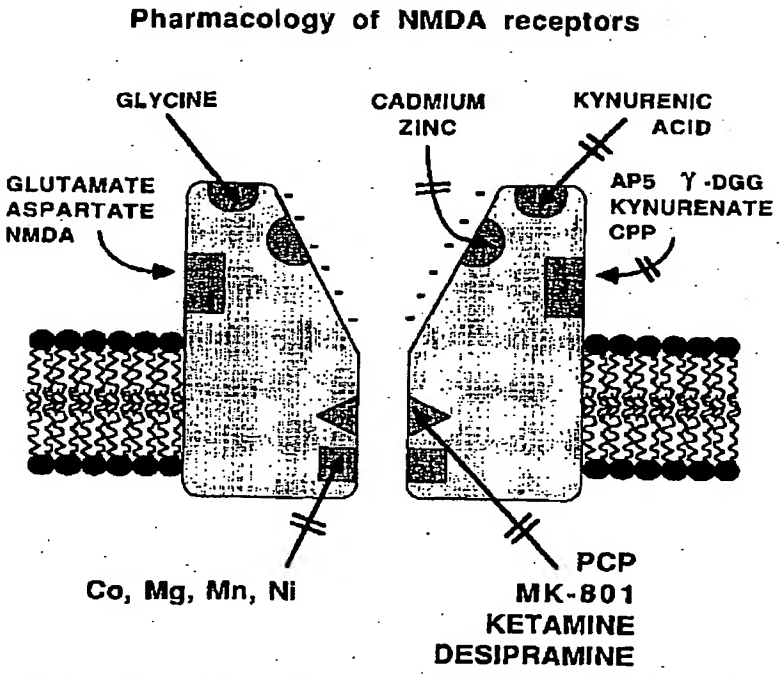

Fig. 8. Multiple sites for drug action at NMDA receptor channels. Three classes of binding site appear to be close to the extracellular face of the membrane. These are 1) the agonist recognition site at which glutamate, aspartate, and NMDA bind to open the channel, and at which the glutamate analogues AP5 (2-amino-5-phosphonovaleric acid), γ-DGG (γ-D-glutamylglycine), kynurenic acid, and CPP {(3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid} act as competitive antagonists; 2) a binding site at which glycine, D-serine, and D-alanine act as allosteric modulators, and at which kynurenic acid and HA-966 (3-amino-1-hydroxypyrrolidone-2) displace glycine binding competitively, producing noncompetitive antagonism of responses to NMDA; 3) a binding site at which zinc and cadmium act as noncompetitive antagonists. Two additional binding sites appear to be located in the pore of the channel; these are 1) a binding site for Mg and related divalent cations, and 2) a binding site for dissociative anaesthetics. Because these binding sites are within the membrane electric field, the membrane potential strongly influences the dissociation constant of these ions and drugs, which when they bind block ion flux through the channel.

continuation of that program; studies using Arsenazo III were performed in the laboratory of Dr. A.B. MacDermott. We thank Drs. S. Cull-Candy, J. Huettner, L. Nowak, and P. Schwindt for permission to illustrate results from papers they have published.

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